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13. ABSTRACT *(Maximum 200 Words)*

The existence of prostate stem cell capable of giving rise to all the epithelial lineages present in the adult prostate is very controversial. Understanding the stages of cell differentiation in normal prostate epithelium is essential for the identification of the cell type(s) involved in prostate carcinogenesis. The p53-homologue p63 is selectively expressed in the basal cell compartment of a variety of epithelial tissues and p63 deficient mice show severe defects in the development of epithelial organs, including agenesis of the prostate. These findings suggest that p63 is required to maintain a prostate stem cell population. In order to test this hypothesis we will first study p63 expression in the various stages of prostate development in wild type mice by both immunohistochemistry and in situ hybridization (Specific Aim 1). We will also construct chimeric mice by injecting p63+/+ β-galactosidase positive ES cells into p63-/- blastocysts (Specific Aim 2) and then analyze the relative contribution of p63+/+ and p63-/- cells to the prostatic epithelium. In the event in which both basal and secretory cells require p63 for development, the results will indicate that both compartments originate from a common p63-positive stem cell.

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INTRODUCTION

One substantial limitation in understanding the molecular events that lead to prostate cancer is that the cell type undergoing neoplastic transformation in the prostate is unknown. Moreover, although three major cell types have been identified within the prostate epithelium, the hierarchical relation between them remains obscure and the existence of prostate stem cells is uncertain (1-6). It appears clear that unraveling the epithelial hierarchy in the normal prostate epithelium has important implications in identifying the cell of origin of prostate carcinoma and its pathogenetic mechanisms. The aim of this proposal is to identify prostate stem cells.

The p53 homologue p63 is selectively expressed in the basal cell compartment of the several epithelia, including the prostate. p63 knock-out mice show severe defects in the development of epithelial organs, including the agenesis of all squamous epithelia, breasts, salivary glands and lachrymal glands (7,8). We have recently demonstrated that do not develop the prostate (9). These findings imply that during embryogenesis p63 is required to maintain an epithelial cell population that plays a crucial role in prostate morphogenesis. Two main hypotheses can explain the defect in prostate development in p63-/- mice: 1) p63 is essential for maintaining a prostate epithelial stem cell population that generates both basal and secretory cells 2) p63 is essential for maintaining prostate basal cells which do not represent prostate stem cells but are essential for prostate development. In order to test these hypotheses we are constructing chimeric mice by injecting p63+/+ β-galactosidase positive ES cells into p63-/- blastocysts. If, as expected, p63+/+ ES cells abrogate the defect in prostate development, we will analyze the relative contribution of p63+/+ and p63-/- cells to the prostatic epithelium in rescued chimeric mice. This proposal represents a unique approach to resolve the long-standing controversy on the role of prostate basal cells as stem cells. This chimeric model, if successful, may be applied as an innovative approach to study prostate development and neoplastic transformation in vivo. By utilizing genetically altered β-galactosidase positive ES cells (e.g. Rb-/- or p53-/-) it will be possible to generate mice that carry specific genetic alterations targeted to prostate epithelial cells and investigate their role in tumorogenesis and tumor progression.

Body

Research accomplishments based on the approved Statement of Work

Aim 1. To assess the distribution of p63 positive cells in the developing prostate.
The goal of this specific aim is to confirm that the absence of p63 causes arrest in prostate morphogenesis at the stage of budding from the urogenital sinus by comparing prostate development in wild type and p63-/- mice with a detailed morphologic analysis. In addition, by utilizing immunohistochemistry and in situ hybridization, I plan to determine if all cells in the early prostatic buds of wild type mice are p63 positive.
In this first year of work, 20 p63+/- female mice were mated with 20 p63+/- males and pregnant females were sacrificed at 17, 18, 19 and 20 dpc to obtain embryos at different stages of development. Normally developed embryos as well as mutant ones (p63-/-) were sectioned, fixed in 10% buffered formalin, de-hydrated and embedded in paraffin for histological, immunohistochemical and in situ hybridization studies. In order to optimize the in situ hybridization methodology for the detection of p63 transcripts, a fragment of p63 mouse cDNA (common to all p63 isotypes) was subcloned in pBluescript II KS (Stratagene). In situ hybridization experiments are currently being performed utilizing skin and prostate from wild type mice as positive controls.

Aim 2: To determine which cell compartment(s) require(s) p63 expression for normal prostate development in the mouse.

In order to assess whether or not the p63 positive epithelial cells in the developing prostate represent prostate stem cells that are able to differentiate into both basal and secretory cells, I am utilizing a chimeric mouse system. My aim is to abrogate the defect in prostate development associated with p63 deficiency by injecting p63+/+ ROSA26 ES cells into p63-/- blastocysts and then analyze the relative contribution of p63+/+ and p63-/- cells to the prostatic epithelium of rescued chimeras. In the first year of work the following tasks have been completed:

1. Derivation of the virus-free p63 colony and generation of MPF (mouse pathogen free) p63 colony
1 male and 1 female p63+/- were received from Frank McKeon (Harvard Medical School, Boston MA). This breeding pair was utilized to generate a virus free foundation colony that was then expanded in order to obtain the number of animals necessary to perform the first blastocyst complementation experiment (see #4). Meanwhile, in order to avoid future infection or contamination of the animals, the mouse colony was rederived and brought into a pathogen free barriered facility. I have now expanded the MPF colony (10 mating trios) and discarded the original virus-free colony.

2. Backcrossing 129S4;Balb/c p63 mice into C57Bl6
The contribution ES cells in blastocysts complementation experiments is usually significally higher when performed in C57Bl6 background as compared to mixed background. In order to optimize these experiments, I have therefore decided to introgress (genetically transfer) the p63 gene distruption from 129S4;Balb/c onto C57Bl6

genetic background. To this end I have been utilizing a marker assisted selection protocol (speed congenics) that allows the construction of congenic strains in about half the number of generations required by the classic protocol (N5 versus N10). The N5 generation has been recently obtained, rederived and brought into a pathogen free barriered facility

3. Assessment of beta-galactosidase activity in mouse prostate of Rosa26 and wild type mice

Rosa26 mice have been reported to ubiquitously express beta-galactosidase. However, beta-gal activity has never been assessed at a cellular level in the prostate. Therefore, we performed X-gal histochemistry in different regions of the prostate of three Rosa26 mice. Interestingly, we observed that beta-gal activity was homogeneously detected in both basal and secretory cells as well as in stromal cells exclusively in the ventral prostate. In contrast, in the lateral, posterior and anterior prostate beta-gal activity was not detected in a consistent percentage of luminal cells. As a result, only the ventral prostate appears suitable to evaluate the results of blastocyst complementation experiments utilizing Rosa26 ES cells. As expected, X-gal staining of all prostate regions from wild-type mice did not show any beta-galactivity.

4. Subcloning Rosa 26 ES cells and generation of chimeras by injecting wild-type C57Bl6 blastocysts

Rosa26 ES cells were obtained from Stuart Orkin (Children's Hospital, Boston MA). The ES cells were cultured on a feeder layer of mitotically inactivated (by irradiation) STO fibroblasts. Colonies showing the best morphologic features (i.e. absence of any overt differentiation) were isolated and subcloned. In order to assess the efficiency of the Rosa26 ES cells in contributing to the chimeras, three different subclones were injected in 3.5dpc blastocysts isolated from wild type C57Bl6 pregnant mice. The embryos were then transferred to 3 foster mothers. The degree of chimerism of the chimeric mice was assessed by both color coat and X-gal histochemistry of skin, intestine, liver and prostate. The Rosa 26 ES clone generating the higher levels of chimerism (ranging from 40 to 90% in the various animals) was selected for complementing the p63-/- blastocysts (see #5).

5. Complementing p63-/- blastocysts with Rosa26 (p63+/+) ES cells

Generation of chimeric mice

Two separate experiments have been completed. A third blastocyst complementation experiment has been performed and the offsprings will be born shortly.

Experiment 1: Ninety-four female mice were generated by a peak set up and fourty-four were proved to be heterozygous (p63+/-) by PCR genotyping. At 4 weeks of age the 44 heterozygous females were injected with PMS (5IU). After 47 hours 5IU of hCG were administered to the mice. Immediately after the administration, the mice were placed in cages with fertile heterozygous males (one female for one male). The following morning, the females (0.5 dpc) were examined for the presence of vaginal plugs. In 35 female mice vaginal plugs were detected. 11 mice were utilized for blastocysts isolation. 74 blastocysts (selected from a total of 200 isolated blastocysts), were injected with the previously selected subclone of the Rosa26 ES cells (see above). Embryos were than

transferred to 10 foster mothers. The foster mothers gave birth to a total of 12 male pups which appeared grossly normal. 3 mice died and the remaining 9 mice have been analyzed at 4 months of age.

Experiment 2: Thirteen p63+/- female mice were superovulated and mated to p63+/- male mice as described above. Seventy blastocysts isolated from 10 plugged females were injected with Rosa26 ES cells and transferred to 5 foster mothers. Mutant p63-/- mice are difficult to detect after birth because they are rapidly eliminated by the mother. Therefore, in order to check for the presence of possible non-rescued or incompletely rescued p63-/- embryos, one foster mother was sacrificed at 18.5 dpc. A second foster mother gave birth to 8 male pups (4 of which died immediately after birth). The remaining 3 foster mothers did not deliver and were sacrificed at 20.5 dpc. One of them carried 10 dead embryos that were at different stages of decomposition and appeared normal when evaluated for the presence of limbs. One mother carried only one embryo characterized by the p63-/- phenotype while the remaining mother did not carry any embryo. A total of 17 embryos (18.5 or 20.5 dpc) were processed and analyzed. Only three pups survived and will be analyzed at 4 months of age.

Analysis of chimeric mice

Firstly, chimeric mice were analyzed by X-gal histochemistry in order to assess the degree of chimerism in organs that do not require p63 for normal development, namely the intestine and liver. Since the liver of 18.5 and 20.5 embryos contains a considerable amount of hemathopoietic cells, which express relatively low levels of beta-galactosidase in Rosa26 mice, only the intestine was evaluated in this subset of mice. As a second step, the level of chimerism was assessed in the epidermis and prostate/urogenital sinus epithelium, both of which require p63 expression for normal development. In all X-gal histochemistry experiments tissue sections from Rosa26 and wild-type mice were utilized as positive and negatice controls, respectively. Results are summarized in Table 1. An example of X-gal staining of ventral prostate tissues is shown in Fig.1.

In order to identify chimeras originating from p63-/- embryos, skin samples stained with X-gal were subsequently immunostained for p63. Results showed that in all the samples in which the epidermis was composed of both beta-gal positive cells (derived from Rosa26 ES cells) and Beta-gal negative cells (derived from the original embryo), the negative cells expressed p63. These data indicate that this group of chimeras derived from either p63+/- or p63+/+ embryos. We did not observe any case in which p63-/- cells contributed to normally developed epidermis. This result suggests that p63 function is cell autonomous. Three mice were exclusively composed of Rosa26 cells while two additional ones did not show any contribution by Rosa26 cells (one presented a p63-/- phenotype). Interestingly the epidermis, prostate (both basal and secretory cells) or urogenital sinus epitheliium of four mice were exclusively composed by Rosa26 cells, while intestine and/or liver demonstrated varying degrees of chimerism (60%-95%). This subset could represent p63-/- rescued mice. I am currently working on the development of a methodology that will allow the identification of the genotype of the original embryos in these potentially rescued mice. Two approaches are possible:

a. isolation of beta-gal negative cells from chimeric tissues by laser capture microdissection and genotyping by PCR

b. identification of polymorphisms able to distinguish between the original p63 mouse strain and the Rosa26 ES cells strain, localized in the region of the p63 gene deleted in p63-/- mice. PCR amplification of such polymorphisms will allow the identification of the chimeras derived from p63-/- embryos by demonstrating the absence of the polymorphism characteristic of the original mouse strain.

Table 1. X-gal histochemistry was performed on frozen tissue sections from liver (two samples), skin (two samples), intestine and prostate/urogenital sinus of chimeric mice. The percentage of epithelial cells positive for beta-gal activity is reported for each sample.

Mouse #	Age	Phenotype Sex	Liver #1	Liver #2	Intestine	Skin #1	Skin #2	Prostate/ UG sinus
1	4 m	WT/ M	<5%	<5%		80%	100%	<5%
2	4 m	WT/M	70%	60%		100%	100%	100%
3	4 m	WT/M	<5%	<5%		<5%	30%	0%
4	4 m	WT/M	50%	30%		100%	<5%	65%
5	4 m	WT/M	70%	65%		15%	15%	15%
6	4 m	WT/M	35%	45%		40%	<5%	<5%
7	4 m	WT/M	80%	80%		90%	90%	100%
8	4 m	WT/M	80%	75%		50%	60%	60%
9	4 m	WT/M	70%	65%		60%	75%	25%
10	18.5dpc	WT/M			15%	25%		0%
11	18.5dpc	WT/M			35%	70%		45%
12	18.5dpc	WT/M			80%	100%		100%
13	18.5dpc	WT/M			35%	85%		15%
14	18.5dpc	WT/M			100%.	100%		N/A
15	18.5dpc	WT/M			65%	100%		N/A
16	18.5dpc	WT/M			80%	100%		65%
17	18.5dpc	WT/M			50%	75%		30%
18	18.5dpc	WT/M			0%	0%		0%
19	18.5dpc	WT/M			85%	90%		80%
20	18.5dpc	WT/M			100%	100%		N/A
21	21.5dpc	WT/M			0%	0%		0%
22	21.5dpc	WT/M			95%	100%		100%
23	20.5dcp	WT/M			50%	55%		55%
24	20.5dcp	WT/M			60%	100%		100%
25	20.5dcp	WT/M			40%	90%		50%
26	20.5dcp	WT/M			100%	100%		N/A

Fig.1. Frozen tissue sections of ventral prostate stained with X-gal. Both secretory and basal cells of a Rosa26 mouse show homogeneous beta-gal activity (*left panel*). Prostate glands of a chimeric mouse consist of both beta-gal positive and beta-gal negative epithelial cells (*middle panel*). No beta-gal activity is detectable in the prostate of a wild-type mouse (*right panel*).

KEY RESEARCH ACCOMPLISHMENTS

Aim 1

a. p63+/+, p63 +/- and p63-/- embryos at 17, 18, 19 and 20 dpc were collected for histological, immunohistochemical and in situ hybridization studies.

b. Working on optimization of in situ hybridization for detection of p63 transcripts in skin and prostate tissue sections.

Aim 2

a. Derivation of the virus-free p63 colony and generation of MPF (mouse pathogen free) p63 colony.

b. Backcrossing 129S4;Balb/c p63 mice into C57Bl6.

c. Assessment of beta-galactosidase activity in mouse prostate of Rosa26 and wild type mice.

d. Subcloning Rosa 26 ES cells and generation of chimeras by injecting wild-type C57Bl6 blastocysts.

e. Generation of chimeras by injecting the selected subclone of Rosa 26 ES cells in blastocysts generated by crossing p63 +/- females with p63+/- males.

f. Analysis of 26 chimeric mice by X-gal histochemistry and p63 immunohistochemistry.

REPORTABLE OUTCOMES

Manuscripts sponsored by the DADM17-01-1-0051 proposal:

1. Neal Lindeman, David Waltregny, **Sabina Signoretti**, Massimo Loda. Gene transcript quantitation by real time RT-PCR in cells selected by immunohistochemistry-laser capture microdissection. Diagn Mol Pathol. In press.

2. Michael H. Weinstein, **Sabina Signoretti**, Massimo Loda. Diagnostic Utilility of Staining for p63, a Sensitive Marker of Prostatic Basal Cells. Manuscript submitted.

3. Levi A. Garraway, Douglas Lin, **Sabina Signoretti**, David Waltregney, James Dilks, Nandita Bhattacharya, Massimo Loda· Intermediate Basal Cells of the Prostate: *In Vitro* and *In Vivo* Characterization. Manuscript submitted.

4. **Sabina Signoretti**, Lucia di Marcotullio, Andrea Richardson, Sridhar Ramaswamy, Andrea C. Carrano, Beth Isaac, Montserrat Rue, Franco Monti, Alberto Ravaioli, Massimo Loda, Michele Pagano. Oncogenic role of the ubiquitin ligase subunit Skp2 in human breast cancer. Manuscript submitted.

CONCLUSIONS

The major achievement is the demonstration that complementation of p63-/- blastocysts by injecting wild-type Rosa26 ES is feasible. I have generated and analyzed 26 chimeric mice, four of which represent potential rescued p63-/- embryos. The analysis included optimization of X-gal histochemistry and subsequent p63 immunohistochemistry on frozen tissue sections. My preliminary results show that the level of chimerism obtained is variable and overall appropriate for the identification of the rescued chimeras. More importantly, these results seem to indicate that p63 function is cell autonomous and therefore confirm the relevance of this animal model in the assessment of the role of p63 positive basal cells as prostate stem cells. I am currently working on the development of a methodology to precisely assess the genotype of the original embryos in the potentially rescued mice. This represents an essential step in the correct evaluation of the chimeras. In conclusion, my future plans include the systematic analysis of the collected tissues for Aim 1. Furthermore, I will perform additional blastocyst complementation experiments and analyze the chimeras at various stages of development (Aim 2).

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